NEURULATION IN MECHANICALLY AND CHEMICALLY INHIBITED AMBLYSTOMA

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Introduction

Although the dependence of the medullary plate upon the chorda-mesoderm has attracted considerable attention from embryologists, the mechanism by which the plate becomes a neural tube has not been demonstrated.

In amphibians, it has been claimed that pressure exerted by ectoderm and mesoderm (Giersberg, 1924) or by the liquid confined between those two germ layers (Ruffini, 1925) is an active factor in neurulation. However, Lehmann (1926) and Boerema (1929), using different experimental approaches, have demonstrated that neurulation in these forms is an autonomous process within the medullary plate. In echinoderms (Moore and Burt, 1939; Moore, 1941) gastrular invagination, which in many respects resembles neurulation, has likewise been shown to be independent of ectodermal pressure.

Mitosis accompanied by a differential increase in cell volume has also been thought to be a factor in neurulation. Although little or no mitotic activity during this process was found by Glaser (1914) in *Cryptobranchus alleghenienis* or by Ruffini (1925) in Triton, the latter worker believes mitosis to be a contributing factor to neurulation in Rana. Derrick (1937) reports that the high mitotic rate in the sides of the chick medullary plate as compared with the floor may aid neurulation in that form. In this animal it has also been found that after the neural tube has closed, incidence of mitosis is higher in the evaginating optic vesicles than it is in other regions of the brain (Frank, 1925). Hutchinson (1940), on the other hand, finds that the elongation of the neural tube which occurs soon after its closure in Amblystoma is not due to cell proliferation.

The hypothesis of Glaser (1914) that neurulation in Cryptobranchus may be caused by differential water absorption in the medullary plate cells has not been supported by the data of Brown, Hamburger, and Schmitt (1941) on Amblystoma. They find no appreciable increase in the water content of the plate during the critical period as determined by density measurements. Hobson (1941), however, was able to produce unfolding of partially closed chick neural tubes by dehydrating them in hypertonic media.

Ruffini (1925) reports that neurulation is aided by autonomous, amoeboid motion of the medullary plate cells. Boerema (1929) concludes that autonomous changes in cell shape are the responsible mechanism. It is well established (Goerttler, 1925; Vogt, 1929; Manchot, 1929, and many others) that extensive

[1] This investigation was carried out under the direction of Dr. Paul Weiss. It forms a part of a thesis on "Chemical Factors in Nerve Development" presented in partial fulfillment of the requirements for the Ph.D. degree. It has been supported by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

cell movements take place within the neural ectoderm which result in the elongation of the structure, but it is not known to what extent these movements are correlated with the formation of the neural tube.

It was the purpose of the work reported here to compare the cellular changes taking place in normal embryos during neurulation with those in embryos in which neurulation had been inhibited by various means in an attempt to find some clue to the factors responsible.

Materials and Methods

Several clutches of eggs of *Amblystoma maculatum* (Shaw) and of *Amblystoma tigrinum* (Green) were used, some of which were obtained near Chicago and some of which were shipped from Pennsylvania. The eggs were reared at room temperature unless otherwise noted, and care was taken that environmental conditions should be the same for experimentals and controls in a given series. Stage numbers of all specimens refer to Harrison's tables (1918, unpublished).

Most of the embryos were fixed in modified Formol-Zenker, double embedded in celloidin and paraffin, and sectioned at 6 micra. Some specimens were stained with Ehrlich's hematoxylin and mucicarmin for the study of cell shape, nuclei, and pigment granules; others were stained with neutral gentian violet to differentiate yolk and secretion granules. A few embryos were fixed in picric alcohol and stained with Best's carmine for the determination of glycogen.

Mechanical Inhibition of Neurulation

Firstly, mechanical inhibition of neurulation was accomplished as follows: The medullary plates plus underlying mesoderm were excised from each of two *Amblystoma maculatum* embryos in Harrison's Stage 12 and explanted into Holtfreter's solution. One plate was then placed on top of the other and the two pieces of tissue weighed down with splinters of cover glass in such a manner that the plates could not fold up to form a tube. In some cases the plates were oriented so that the ectoderm of one was in contact with the mesoderm of the other; in other cases ectoderm was in contact with ectoderm. Six double explants of this type were studied. A number of intact Amblystoma eggs from the same clutch from which the membranes had been removed were reared in Holtfreter's solution, and 10 explanted medullary plates were allowed to develop freely in the same medium as controls.

The unoperated eggs developed normally except that, in some cases, the hypertonic medium caused a slight retardation of the head region. By the time the normal controls had reached Stage 28, the free explants showed distinct signs of neurulation. When the normal controls were in Stage 31 (Plate I, Fig. 1), the free explants had prominent neural folds which in some cases had nearly closed to form a tube (Plate I, Fig. 2). At the same time in the weighted explants, the medullary cells had elongated and become columnar as in early stages of normal neurulation, but the flask shape characteristic of later stages was never assumed and a tube was not formed (Plate I, Fig. 3).

There was no apparent difference in cell shape or intracellular organization between weighted explants whose ectoderm was in contact with ectoderm and those whose ectoderm was in contact with mesoderm. Thus it would seem that

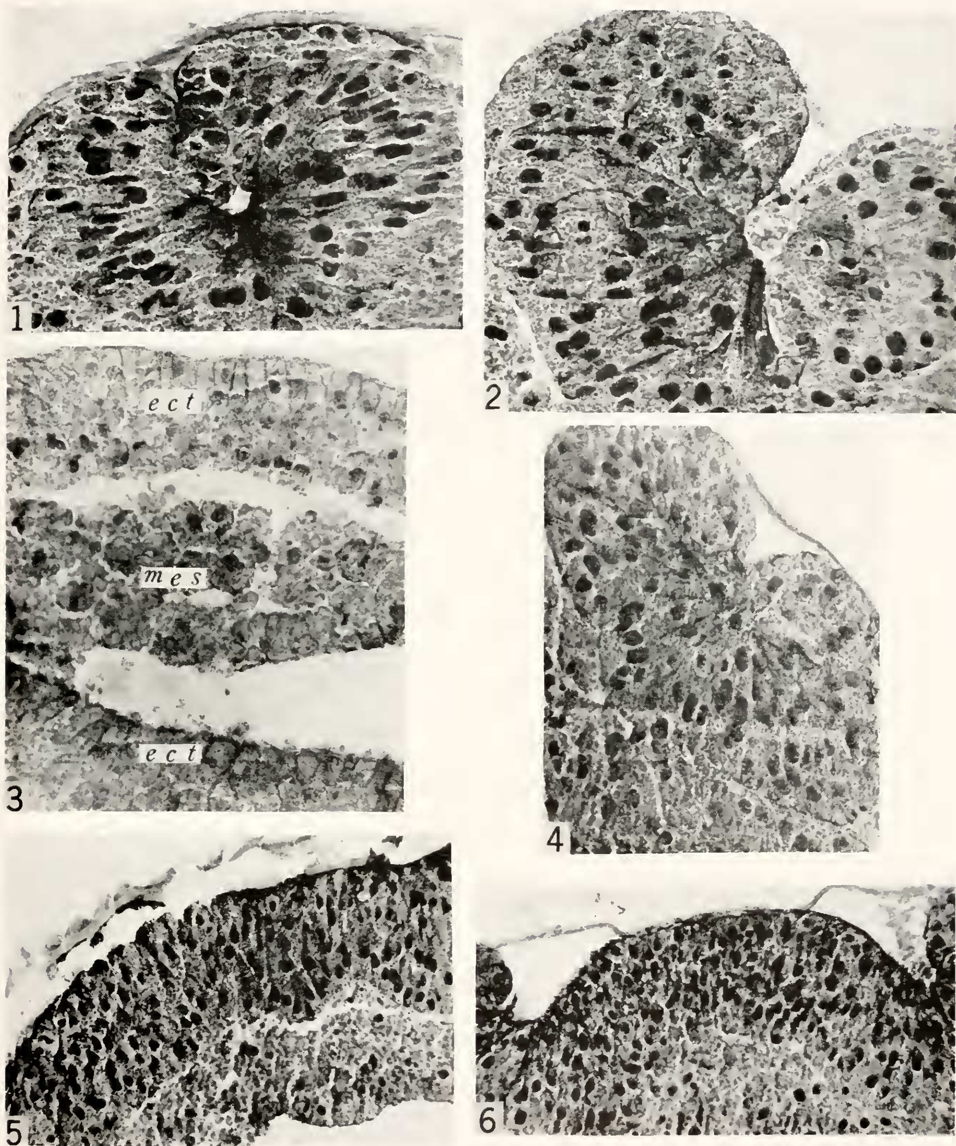


PLATE I

FIGURE 1. Neural tube of normal *A. maculatum* embryo. 250 ×.

FIGURE 2. Medullary plate from embryo of same chronological age as Figure 1 explanted into Holtfreter's solution. 250 ×.

FIGURE 3. Double explant, same age as Figure 1. ect. = neural ectoderm. mes. = mesoderm. 250 ×.

FIGURE 4. Normal *A. tigrinum*, Stage 18. 250 ×.

FIGURE 5. Ringer-treated embryo, same chronological age as Figure 4. 160 ×.

FIGURE 6. LiCl-treated embryo, same chronological age as Figure 4. 160 ×.

by Stage 12 the dorso-ventral polarity of the neural plate has already been established. In both the weighted and free explants the cells were rounder and shorter than those in the controls, the nuclei were round as compared to the oval ones in the normal animals, and there was a heavier deposit of pigment granules around the distal edges of the medullary cells. No other significant differences were noted.

From these data it was concluded that, while pressure at right angles to the plane of the medullary plate can inhibit closure of the neural folds, it does not suppress the initial cell elongation which accompanies that closure.

Chemical Inhibition of Neurulation

Next the developing eggs were subjected to the action of lithium chloride and of hypertonic salt solutions which, in the proper concentrations, will produce delayed closing of the neural tube or permanent *spina bifida*. Three series of experiments were carried out.

Table I

Comparison of development of normal, LiCl- *and Ringer-treated Amblystoma. Figures refer to Harrison's Stages*

	Normal controls	M/10 LiCl	Mammalian Ringer's	Remarks
Series 1				
A. tigrinum	19	15	16	
20° C.	29–30	16	18–19	
	34–35	18–19	20–21	neural tube still open in head region
Series 2				
A. maculatum	16–18	13	12	
12° C.	19–20	15	12	
	22–23	18–19	—	died about 140 hours after immersion in salt solution

The first series consisted of three groups of 33 *Amblystoma tigrinum* eggs which at the inception of the experiment were in Stage 13. The first group were reared in well water to serve as normal controls. The second group were reared in M/10 LiCl solution, the third in mammalian Ringer's. A second series consisted of three groups of 17 *A. maculatum* eggs which at the beginning of the experiment were in Stage 12 b. As with the *tigrinum* eggs, one group was reared in well water, one in M/10 LiCl, and one in mammalian Ringer's. However, the *maculatum* eggs, instead of being kept at room temperature, were placed on a water table with a practically constant temperature of 12° C.

The nervous systems of the treated animals in both series diverged considerably from the mean of normal development. In general, the head region was more retarded than the spinal cord. The approximate degree of maturity attained by the experimentals in comparison with the controls is shown in Table I. In staging the treated animals, external appearance was the criterion used.

It should be noted that the difference between the normal and lithium-treated embryos is greater at 20° C. than at 12° C. (this confirms the work of Hall (1942)

on *Rana pipiens*) but that low temperatures apparently augment the effect of Ringer's solution.

The third series consisted of three groups of 17 *A. tigrinum* eggs which were in Stage 11 b—12 a at the beginning of the experiment. One group served as normal controls, one group was immersed in M/20 LiCl for 24 hours, after which development was allowed to continue in well water, and the third group was similarly treated with M/20 NaCl. NaCl treatment had no perceptible effect on the rate or type of development, while the equimolar LiCl solution retarded the embryos considerably. This series of eggs was fixed in picric alcohol for a rough determination of glycogen content.

Effects of chemical inhibition on mitotic rate

The effects of chemical inhibition were best seen in the first series of eggs as the Ringer-treated eggs did not develop at all in the second series. One-third of

TABLE II

Mitotic rate in the medullary plate of A. tigrinum. Stage numbers not in parenthesis refer to normal controls; those in parenthesis refer to inhibited animals of the same chronological age as the normal controls

	Stage	Cells counted	Mitoses seen	Mitotic index
Normal controls	18	1461	39	2.67%
	30	776	29	3.73%
	35	1037	42	4.05%
Lithium chloride-treated	18(15)	1970	19	0.96%
	30(16)	1109	12	1.08%
	35(18)	693	3	0.43%
Ringer's treated	18(16)	2276	28	1.23%
	30(18)	760	18	2.37%
	35(20)	1074	30	2.79%

the embryos were fixed and sectioned when the normal controls were in Stage 18 (at which time the normal germs had open medullary plates with well raised neural folds), one-third when the controls were in Stage 30, and the remainder when the controls were in Stage 35, by which time the lithium embryos were in approximately the same stage of development as the controls at Stage 18 as far as external appearance was concerned, and the Ringer-treated germs were slightly more mature. The effect of this inhibition on the mitotic rate in the medullary plate is summarized in Table II.

From this it is apparent first, that there is mitosis in the neural plate of *A. tigrinum* during neurulation; secondly, that the mitotic rate rises in the normal animal after the neural tube is closed; thirdly, that Ringer's solution depresses the mitotic rate in comparison with normal embryos of the same chronological age, but that the mitotic rate in Ringer-treated animals is comparable to that in normals of the same stage of development, and fourthly, that LiCl causes both a relative and an absolute decrease in the mitotic rate of the neural tube.

Effects of chemical inhibition on cell shape

The effects of inhibition on the cellular morphology of the neural tube were extreme. When the normal controls were in Stage 18 (Plate I, Fig. 4), the Ringer-treated animals showed a slight evagination of the floor of the medullary plate (Plate I, Fig. 5), and lithium-treated embryos a very marked evagination (Plate I, Fig. 6).

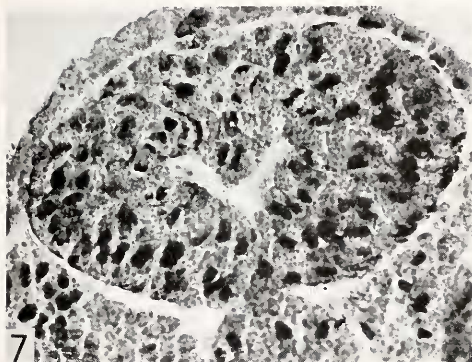
By the time the normal controls were in Stage 30 (Plate II, Fig. 7), ectoderm had begun to grow over the edges of the plate in the Ringer-treated germs and a slight invagination of the plate was present (Plate II, Fig. 8). When the controls were in Stage 35 (Plate II, Fig. 10) and the Ringer-treated embryos in what corresponded to Stage 20 in the normal animals, the invagination was fairly deep in the treated germs and the edges of the plate were raised, although they were not bent over as normal neural folds are at that time (Plate II, Fig. 11).

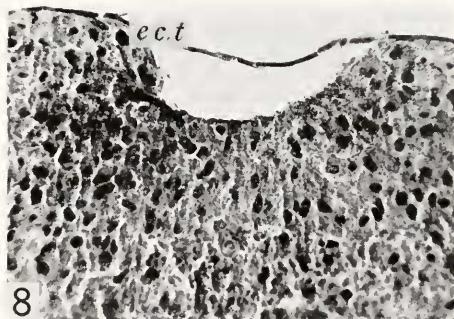
In the LiCl-treated germs, on the other hand, when the controls were in Stage 30, a flat plate was present (Plate II, Fig. 9). When the controls had reached Stage 35 and the lithium-treated animals were in Stage 18 as far as external appearance was concerned, the neural plate was still flat, but a few flask-shaped cells had appeared at the edges as in the first stages of normal neurulation. Many of the medullary plate cells in these embryos, particularly in the head region, became round and sloughed off into the space above the plate (Plate II, Fig. 12). Child (1941) reports a similar dissociation of the endodermal plate in the starfish, Pateria, when exposed to the action of lithium chloride.

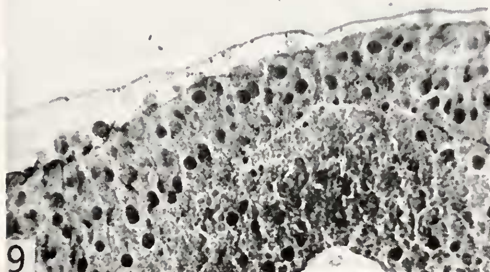
The changes in shape occurring in both the normal and treated embryos naturally correspond to the changes in the shape of the plate as a whole. These changes may be summarized by saying that both Ringer and LiCl treatment produce, first, a more or less evaginated medullary plate and then a flat or slightly invaginated plate which may, according to the concentration of the chemicals used, proceed to form a tube in places or to be overgrown by ectoderm, and that no traces of a neural keel as described by Baker (1927) were seen in the treated embryos in the series studied.

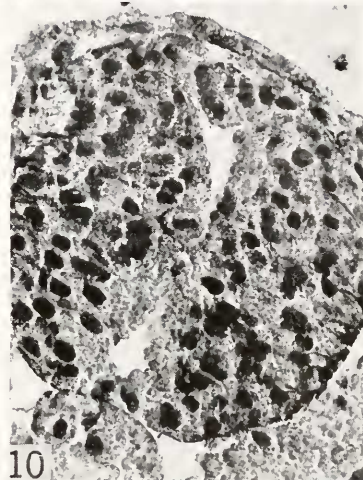
Effects of chemical inhibition on nuclear size

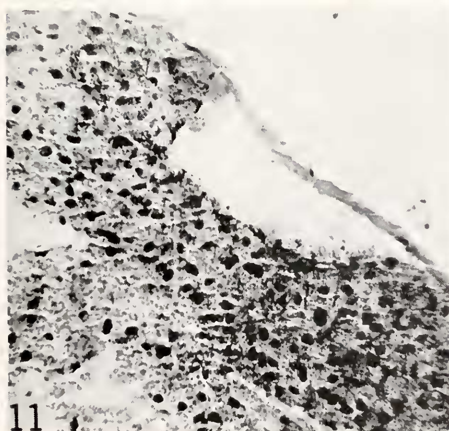
Much importance has been attached to changes in cell and nuclear size during neurulation since Glaser (1914) found that, in Cryptobranchus, the volume of the neural plate increased during the course of neurulation and believed that this indicated an increasing water content of the neural plate. He also inferred that increased hydration occurs during gastrulation in echinoderms because of reported increases in nuclear size during that process. As Brown, Hamburger, and Schmitt (1941) found no indications of increased hydration in density measurements on Amblystoma, an effort was made to throw more light on the problem by measuring the nuclear axes of 100 medullary plate cells in both normals and experimentals in each of three stages. As these nuclei are not perfect spheres and as their orientation varies somewhat within the plate, these measurements cannot be used to calculate nuclear volume. However, any large changes in nuclear volume should be revealed by this method. Indices of nuclear area and shape were also calculated. These data are summarized in Table III.











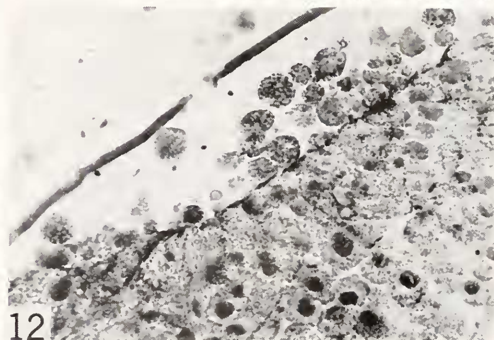


PLATE II

FIGURE 7. Normal *A. tigrinum* embryo, Stage 30. 250 ×.

FIGURE 8. Ringer-treated embryo, same chronological age as Figure 7. ect. = ectoderm growing over medullary plate. 160 ×.

FIGURE 9. LiCl-treated embryo, same chronological age as Figure 7. 160 ×.

FIGURE 10. Normal *A. tigrinum* embryo, Stage 35. 250 ×.

FIGURE 11. Ringer-treated embryo, same chronological age as Figure 10. 160 ×.

FIGURE 12. LiCl-treated embryo, same chronological age as Figure 10. Note round cells sloughed off from plate. 250 ×.

No statistically significant differences in nuclear axes, area, or shape were revealed by this analysis between normal and treated nuclei because of the large standard deviations involved. However, it should be noted that the index of shape (A/B) increased consistently in the normal germs whereas it remained practically constant or decreased slightly in the chemically treated germs. This lack of nuclear elongation seems to be correlated with the failure of cell elongation which was also observed in these cases. While no conclusions can be drawn from these findings as to cellular hydration, there is no change of nuclear size during folding of the neural plate.

Effects of chemical inhibition on cellular inclusions

A. *Yolk granules.* In normal *A. tigrinum* and *A. maculatum* embryos, yolk begins to be utilized in the neural tube, beginning in the head region, about

TABLE III

Greatest nuclear length (A) *and diameter* (B) *of 100 medullary plate cells. Stage numbers not in parenthesis refer to normal controls; those in parenthesis refer to treated animals. All measurements are in ocular micrometer units*

	Stage	Nuclear measurements				Indices			
		Length (A)		Diameter (B)		Area (AB)		Shape (A/B)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Normal controls	18	15.00	2.22	8.84	1.58	129.4	34.8	1.72	0.35
	30	14.60	2.76	8.42	1.56	119.2	30.6	1.80	0.51
	35	16.80	2.83	8.86	1.52	146.6	36.8	1.93	0.48
Ringer-treated	18(16)	13.55	2.41	8.66	1.56	115.9	33.4	1.59	0.38
	30(18)	14.33	2.24	9.03	1.71	126.7	38.8	1.58	0.36
	35(20)	13.27	2.18	9.42	1.56	121.7	32.2	1.42	0.32
LiCl-treated	18(15)	14.79	2.21	9.51	1.46	139.7	34.4	1.45	0.27
	30(16)	14.63	2.22	10.79	1.56	157.3	38.0	1.26	0.25
	35(18)	13.96	2.03	10.02	1.17	138.7	32.0	1.38	0.20

Stage 18. By Stage 35, yolk has practically disappeared from the brain, and only a sparse scattering of granules remains around the lumen of the spinal cord. Bragg (1939), who has studied the utilization of yolk in a number of other amphibian genera, reports that in his animals it did not begin until after the closure of the neural tube.

Lithium chloride and Ringer's solution both seem to retard the disappearance of yolk as well as the closure of the neural tube, as the medullary plates of the treated animals were packed with yolk granules throughout the period of observation whereas, in the normal controls, the amount significantly diminished. It is doubtful if this is causally related to the process of neurulation, however, because (1) yolk disappears very late in this process and (2), as Morgan (1906) has shown, eggs of *Bufo variabilis* centrifuged so that all granules are thrown out of portions of the head in the resultant embryos will develop closed neural tubes.

B. *Glycogen.* In the *A. tigrinum* series, no perceptible change in the glycogen content of the nervous system was noted between Stage 18, at which time the neural tube is open over its full length, and Stage 30, when the entire tube is closed and morphogenesis of the brain is well under way. All the neural cells contained much glycogen, no significant differences being noted among the various regions of the nervous system.

Treatment with M/20 NaCl did not affect glycogen distribution (as was to be expected since no morphological changes were observed) nor did treatment with M/20 LiCl. Thus, although no histological method is exact enough to reveal very small changes in glycogen content, it would appear that in *A. tigrinum* neurulation is not accompanied by significant utilization of this material.

C. *Pigment granules.* Early in the normal process of neurulation in Amblystoma, as reported by Ruffini (1925) and Lehmann (1926) for other urodeles, there is a marked accumulation of pigment, especially in creases formed by the medullary folds. From the pigment layer at the distal ends of the cells, rows of pigment granules extend along the cell boundaries (see Plate I, Fig. 1). When neurulation is completed, there is a layer of pigment granules along both surfaces of the neural tube and many granules along the cell boundaries and within the cell bodies.

The chief difference noted in the treated animals was that the concentration of pigment near the outer surface of the plate cells occurred irregularly and only in those cells where shrinkage of the inner surface took place (see Plate I, Figs. 5 and 6; Plate II, Figs. 8 and 12). In the lithium-treated specimens, as soon as degeneration of the plate commenced, many granules escaped into the free space above the plate, and all of the sloughed-off cells were packed with pigment. These granules, like yolk, however, appear to be a passive factor in neurulation and are of value only as an indicator of the results of active processes which change cell shape.

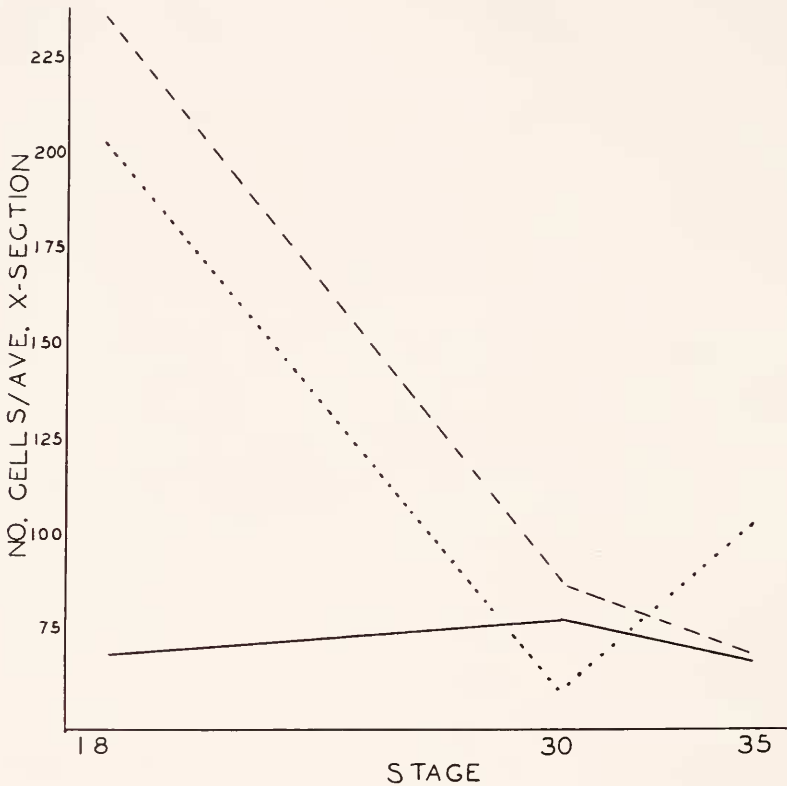
D. *Secretion granules.* As shown by Studnička (1900) and Weiss (1934), secretion occurs in the embryonic ependyma. On the chance that secretion might be involved in the process of neurulation, normal and chemically inhibited *A. tigrinum*, *A. maculatum*, and *Rana pipiens* germs and normal chick embryos which had been stained with neutral gentian violet were examined for secretion granules. None were found, either in the normal embryos or in the treated amphibians. This does not necessarily indicate that unformed secretion does not occur; in fact, the presence of liquid within the lumen of the neural tube is evidence that secretion of some sort does take place very early in normal development. Because of the difficulty of demonstrating secretion antecedents, however, the problem requires study by more refined techniques.

Effects of chemical treatment on cellular movements

The most accurate method of following cell movements in embryonic development is by vital staining, a procedure not used in this investigation. However, some indication of those movements was obtained by counting the number of cells in the neural plate in every fifth section of the trunk region of embryos (exclusive of brain and tail) and averaging the results. It is very hard to obtain strictly comparable results by this method because, as Manchot (1929) has shown, in the normal development of urodele embryos, the anterior two-thirds of the neural

plate becomes brain while the posterior one-third elongates to form the spinal cord. A rough comparison between the normal and chemically treated neural plates at various stages of development is presented in Graph 1.

As this graph was constructed from data on only nine animals, not too much significance can be attached to it. However, it would seem that in normal animals between Stages 18 and 35 mitosis and stretching of the neural plate keep pace with one another so that the average number of cells per cross section remains



GRAPH 1. Average numbers of cells present in every fifth section of the medullary plate of normal and chemically treated *A. tigrinum* embryos of the same chronological age. Abscissae are stage numbers of normal controls. Solid line used for normal controls, dashes for lithium embryos, dots for Ringer germs.

approximately constant and that, although LiCl and Ringer's solution do inhibit the stretching process just as they inhibit neurulation and mitosis, elongation of the neural plate continues under their influence.[2]

[2] In Glaser's work on Cryptobranchus (1914), he used the average number of nuclei present per cross-section to test whether or not cell division was occurring. Because the number of nuclei remained approximately constant, as it does in normal Amblystoma during slightly later stages, he concluded that there was little or no mitosis during neurulation. It would be interesting to re-examine his material to see if elongation of the medullary plate played any role in that constancy.

Discussion

In discussing neurulation, it must be borne in mind that the folding of the neural plate is a very complex process involving not only changes in cell size and proportions, but physical and biochemical changes which have, as yet, been little studied. From the foregoing analysis, it can be concluded that certain factors are not involved in the more obvious phases of folding. Thus not only does neurulation occur, as has been previously reported by many workers, in the absence of normal mechanical pressures, but the characteristic preliminary cell elongation takes place when pressure is exerted at right angles to the usual direction of cell movement.

Neurulation seems to be independent of nuclear area, and if, as has been suggested, the latter be accepted as an index of cell hydration, also independent of hydration. For, although treatment with Ringer's solution and LiCl had no significant effect on nuclear area, it did inhibit folding of the medullary plate. On the other hand, nuclear elongation, which accompanies cell elongation in the normal plate, does not occur when folding is inhibited.

Judging by the data on average number of cells per section, the elongation of the medullary plate which normally takes place during and after neurulation is not necessarily correlated with the closure of the neural tube, because, although both LiCl and Ringer's solution do retard the stretching process somewhat, it continues even when neural folds do not form.

Mitosis seems to be either directly instrumental in neurulation or at least under control of the mechanism of neurulation. Thus in the LiCl-treated embryos, in which the mitotic rate fell to a very low value during the experiment, no folds appeared, while the elevation of the sides of the neural plate in both the normal and the Ringer-treated specimens was accompanied by active cell division.

Cell elongation and wedging are unavoidably correlated with embryonic folding, and, as Boerema (1929) and others have pointed out, such changes are theoretically quite sufficient to cause neurulation. As demonstrated by Brown, Hamburger, and Schmitt (1941) differential water absorption cannot account for such changes in Amblystoma. These workers and Schmitt (1941) independently have suggested that molecular interactions and desolvations in the cell surface may exert the forces necessary to cause cell elongation. Weiss (unpublished) has suggested further that the concentration of pigment granules which occurs in the normal folding plate indicates a contraction of the cell cortex at the free surface. Although Hobson (1941) has not succeeded in demonstrating any systematic changes in the ultrastructure of the chick neural plate during folding by polariscopic analysis, a more intensive investigation of such changes during neurulation appears to be the most promising method of approach to the problem.

An interesting point which emerges in a comparison of the LiCl and Ringer-treated germs is that the effects of the two agents on neurulation seem to be produced by different means. Thus lithium is less effective at low temperatures, while hypertonic salt solutions are more effective. Further, lithium salts inhibit neurulation at much lower concentrations than do those present in Ringer's solution. Hall (1942) has evidence that lithium is a toxic agent acting on the chordamesoderm rather than on the responding ectoderm. The fact that Ringer's solution is a more effective inhibitor at low than at high temperatures would suggest that it acts on the physical consistency of the embryo rather than on chemical

processes, perhaps by stiffening the neural plate so that folding is impeded—a suggestion which Giersberg (1924) has previously offered to explain the action of sucrose and sodium acetate on neurulation.

Finally, it should be noted that the data presented here are by no means conclusive in themselves; they are offered merely in an effort to shed light on a few phases of a very complicated problem.

Summary

1. Mechanical pressure exerted at right angles to the plane of explanted medullary plates has been found to suppress neurulation in *Amblystoma maculatum*, but not the preliminary cellular elongation which is normally involved in that process. This elongation takes place irrespective of whether the medullary plate is in contact with ectoderm or with mesoderm on the normally free surface.

2. In *Amblystoma tigrinum* and *A. maculatum* neurulation is accompanied by mitosis, the mitotic rate rising after the neural tube has closed. Treatment with mammalian Ringer's solution at room temperature decreases the mitotic rate to about the same degree as it inhibits normal development; treatment with M/10 LiCl decreases the mitotic rate both relatively and absolutely.

3. No statistically significant difference was found in average nuclear area between normal and treated medullary plates. In normal germs, the nuclei elongate during neurulation, whereas in the treated germs they did not.

4. Glycogen and yolk begin to disappear from the normal neural tube about Stage 18. Neurulation-inhibiting chemicals retard the utilization of these substances.

5. Pigment granules appear to be passive factors in neurulation indicative of contraction at free cell surfaces.

6. No evidence of formed secretion from the neural plate was found.

7. Although inhibiting chemicals decrease the rate of elongation of the medullary plate, stretching continues even when neural folds fail to form.

8. The inhibiting action of LiCl is less effective at low temperatures, that of Ringer's is augmented.

9. It is concluded that neurulation in Amblystoma is autonomous to the medullary plate and may be aided by mitotic activity; changes in nuclear area (which may be indicative of cell hydration), intracellular inclusions, and longitudinal cell movements are not instrumental in the process.

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